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NOVEL TECHNIQUES TO ESTABLISH NEW INSECT CELL LINES

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Summary

The success of insect cell culture is demonstrated by reports of over 500 established cell lines. While established procedures that can be used for developing new cell lines exist, these usually require some fine-tuning for various tissue sources. This paper attempts to depict some of the variations that can be applied.

Key words: invertebrates; cell-line development; tissue source; cell culture.

Introduction

Continuous insect cell lines have been an important research tool since Grace (1962) developed the first insect cell lines from a moth. In the nearly 40 yr since, the field has been quite productive as seen in Fig. 1. A current list, which I assembled from compilations by Hink (Hink, 1972, 1976, 1980; Hink and Bezanson, 1985; Hink and Hall, 1989) and approximately 30 other published reports over the past decade, shows that there are now over 500 established lines. This graph also shows clearly that most of the lines have been from Lepidoptera and Diptera with just over 20% from all other invertebrates. Figure 2 shows tissues that have been used successfully for developing insect cell cultures. These include:

Ovaries: These were the 'first,' and were commonly used throughout the 1960s and 1970s, especially with Lepidoptera.

Embryos: These are the most commonly used source of cells for cultures. The same compilations used to create Fig. 1 reveal that nearly half of all the insect cell lines were initiated from embryos.

Hemocytes: These are 'easy' cells to obtain, but not easy to grow.

Imaginal discs: These are very important tissues in developmental biology because they are developmentally determined to become specific structures, yet consist of undifferentiated cells.

Fat body: This is an important physiological tissue in that it has many functions similar to the mammalian liver. It is also a target tissue for many insect pathogens.

Midgut: This is relevant to pest control and pathology.

Neonate: Newly hatched larvae—while the tissues are generally more developed than those found in embryos, a significant amount of undifferentiated cells are present.

Cuticle/nervous system/endocrine system/muscular system: These are important tissues in the study of pest control, physiology, and pathology. Although they have each been used in primary culture experiments, established cell lines are rare or nonexistent.

Materials and Methods

Although my standard technique (Lynn, 1996) primarily gives details about the initiation of cultures from embryos, similar methods can be followed for developing insect cell lines from any tissue. The basic procedure is:

(1) Disinfect the insect (or eggs) by submersion in 70% ethanol. If the insects live under filthy conditions, this may be preceded by a 3–5-min submersion in 0.05% sodium hypochlorite (=1% chlorine bleach).
(2) Rinse twice with sterile distilled water.
(3) Transfer to complete culture medium containing antibiotics in a sterile Petri dish. *Note* that the use of antibiotics, such as gentamicin sulfate[2] (Sigma, St. Louis, MO), is practical during the primary culture phase of cell lines, but is to be avoided in established lines. Routine use of antibiotics can mask a low level of contamination and lead to resistant strains of bacteria. I commonly switch to antibiotic-free medium after two or three passages.
(4) Using sterile dissecting instruments (Roboz Surgical Instruments, Washington, DC), remove the tissue of interest and transfer it to a new sterile dish with additional medium.
(5) Tease away any contaminating tissues and set the dish aside for a brief period (30 min–2 h) to allow any contaminating cells (such as hemocytes) to diffuse away.
(6) Transfer the tissue of interest to a 35-mm tissue culture dish (Becton Dickinson, Franklin Lakes, NJ) containing a standing drop (0.1–0.2 ml) of medium, and cut it into small pieces with a microscalpel.
(7) Seal the edge of the dish with Parafilm®, place it (along with a small beaker of sterile water) in a plastic box with a tight-fitting lid, and incubate at 17–35° C. The temperature chosen will depend on the insect species and the intended use of the cells. Most insect cells grow optimally at 25–28° C, but the use of lower temperatures can yield cells with specific desired properties (Winstanley and Crook, 1993).
(8) Add an additional 1 ml of fresh medium after 24–48 h.
(9) Add 0.5 ml additional medium at 7–10-d intervals.
(10) When the culture contains more than 2.5 ml medium, replace all but 0.5 ml with 0.5 ml fresh medium. If most of the cells are attached, simply drawing the excess medium into a pipet and transferring it to another container can be done. Even if there are only a few unattached cells, it is useful to save this excess medium by transferring it to another dish or to a tissue culture flask. These secondary cultures will quite frequently yield dividing cells, even if the primary culture does not. If the culture contains many suspended cells, the excess medium should be transferred to a sterile centrifuge tube, and the cells should be pelleted by centrifuging at 50 × *g*, 10–15 min. Decant the supernatant

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[2] Mention of proprietary or brand names is necessary to report factually on the available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

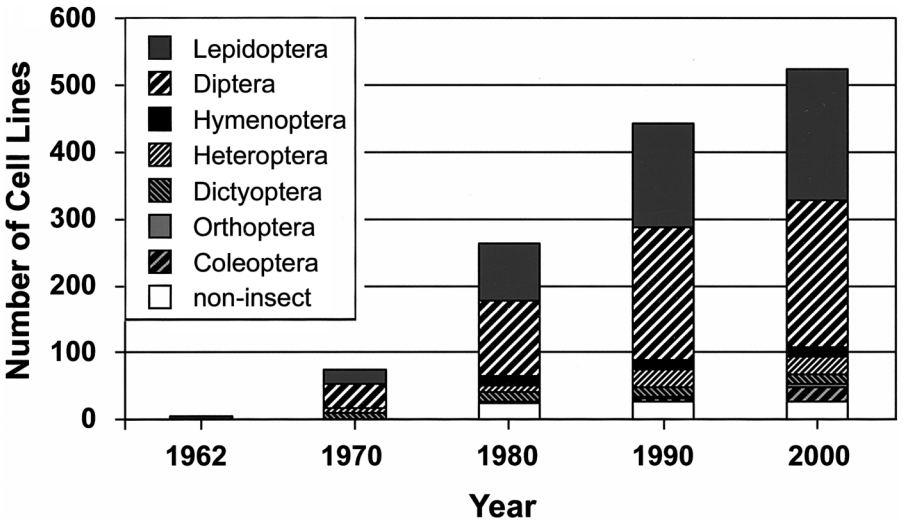

FIG. 1. A number of established invertebrate cell lines developed since 1962, categorized by insect orders. Each *bar* represents the total number of cell lines which had been reported up to the date listed.

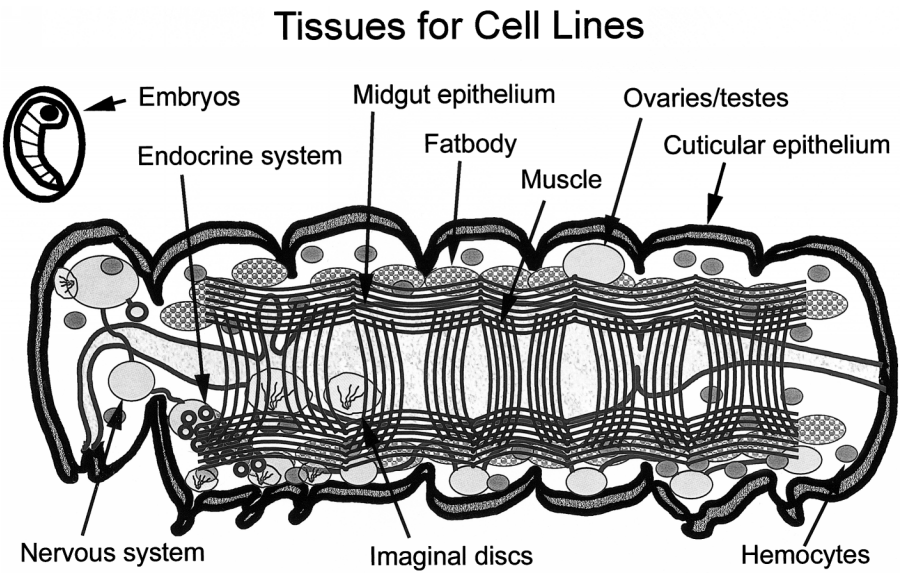

FIG. 2. Diagrammatic representation of an insect showing tissues that can be used for the development of cell lines.

medium and resuspend the cell pellet in 0.5 ml fresh medium, and return it to the primary culture dish.

(11) When the culture is ~90% confluent, subculture the cells at a 1:2 split ratio. A variety of subculture methods can be employed with insect cells. If a substantial number of the cells are suspended or loosely attached, use gentle pipetting to distribute the cells evenly in the medium, then transfer half to a new culture, adding an equal amount of fresh medium to both the original culture and the subculture. If the cells are more firmly attached, they may be suspended by flushing the cell surface with medium from a pipet with more force. Finally, if the flushing technique does not free a substantial proportion of the cells, you can use scraping or enzymatic treatments. *See* Lynn (1996) for the procedures.

Using the above as the basic protocol, the following modifications can be used for application to specific tissues:

Embryos: Since my earlier paper (Lynn, 1996) detailed how to initiate cultures from embryos, this does not require much more explanation, but some modifications can be used which will improve the success rate. For example, if the eggs are particularly dirty, hairy, or have a thick chorion, pretreat with sodium hypochlorite (0.05% for 2–3 min) before using the ethanol to disinfect. Try variable numbers and eggs of different ages to improve the chances of obtaining continuous cell lines.

Ovaries: Again, follow the standard procedure. Use care to clean the ovaries of contaminating tissues, such as fat body, as well as possible, and set up cultures from insects of various developmental stages. Adults, pupae, and larvae have all been successfully used.

Hemocytes: These are easy to obtain. After disinfecting the insect, a leg or proleg can be cut, and drops of the hemolymph collected in a Petri dish with medium. A significant problem with these cells is the melanization of the medium due to phenol oxidase activity in the blood of many insects. Possible solutions are the use of inhibitors, such as reduced glutathione (0.8 mg/ml) or cysteine (0.6 mg/ml), or selecting the larvae at the time of a molt, when they typically have lower phenol oxidase activity.

Neonate larvae: An effective method is to start with the eggs, perform the

disinfection as you would for embryo cultures, but then put the clean eggs at the top of a sterile 15-ml conical centrifuge tube containing 3–4 ml of medium. Usually the eggs that are still wet from disinfecting will stick to the tube wall in this location. The top 10 cm of the tube is wrapped in foil and placed on a tilt with a light source at the bottom. When the eggs hatch, most insect larvae are phototropic and will crawl into the medium, which causes them to drown. After a sufficient number have hatched (this varies depending on the size of the insect; 10–50 is a typical number to strive for), the insects are macerated, and the medium is transferred to a Petri dish or flask. Phenol oxidase can be a problem with whole larvae and can be reduced with inhibitors, as described for hemocytes.

Imaginal disks: These are very good tissue for cell cultures. A significant problem is associated with locating the tissue, since they are commonly small, nearly transparent structures. In other respects, the normal procedure can be used. In some cases, a phenol oxidase inhibitor, such as the one described for hemocytes, may be necessary. An additional problem in some early studies (Lynn et al., 1982) was in recognizing success when it occurred. Imaginal disk cells (as well as other insect epithelial cells) tend to grow as multicellular vesicles, which are considerably different from most continuous cell lines.

Fat body: My personal success is limited to a single line from fat body cells (Lynn et al., 1988), but other researchers have been more successful (Mitsuhashi, 1981, 1983, 1984; Philippe, 1982). These are usually a very plentiful cell type in larvae but may not be commonly primed for cell division. Use standard disinfection and medium, but try different aged larvae, pupae, or adults. Also, if you do not need cells from a particular insect, try different species.

Midgut: This is a very important tissue in insect biology and control but, by its nature, may pose many difficulties for its use in cell culture. For example, it can be contaminated with various microorganisms from the diet of the insect, it produces enzymes that can have a detrimental effect on the cells, and one cell surface may be exposed to a drastically different pH compared to most insect tissues. Some possible approaches include starving the insects to reduce the gut contents or using intermolt insects. Loeb and Hakim (1996) used the following method to obtain high-quality midgut cells for primary cultures: after excision, swirl periodically in medium for 1 h to isolate the stem cells. Add 20-OH-Ec (1 ng/μl) to the medium. Use medium conditioned with other midguts or fat body extract.

Other suggestions. In many primary cultures (especially those set up from embryos, whole larvae, or other variable tissues), many cell types can be seen. These occur often in discrete colonies, which, with some manipulation, can be isolated into separate cell strains by mechanical techniques. Instruments that can be used for isolating such colonies can be fashioned from a standard 200-μl pipet tip. Grab the narrow end of the tip with a pair of forceps, and pull while squeezing. This results in a flattened tip that still has a channel running through it. Cutting the tip with a scalpel or razor blade gives a straight edge which can be used after sterilization to scrape the cells while you suction them into the pipet. Cells collected in this manner can then be transferred to a new culture dish and can develop into a uniform cell line.

Another useful method is to use feeder layers. These can provide growth factors when obtaining cells from small insects or difficult-to-obtain tissues or when previous attempts to develop cell lines with normal methods have failed. Feeder layers are also useful for cloning an established line. For fastidious cell types, you should use feeder layers from (in order of preference based on my experience): (1) the same cell line (for cloning), (2) a cell line from the same species, (3) a cell line from the same order, (4) a cell line from the same tissue, or (5) cells from a fast-growing line on the same medium. A specific technique that I have used for feeder layers is to use inserts which fit into multiwell plates (such as those made by Millipore and Costar). Depending on your cells, you can place the feeder cells either inside the insert, with the cells of interest in the multiwell plate, or in the opposite way.

One other method that can be somewhat useful is the use of low temperatures for maintaining or establishing cell lines. A temperature of 17° C allows many insect cell lines to grow at a slow rate, so subcultures may be made less frequently (monthly). Low-temperature maintenance can also positively affect virus susceptibility (Winstanley and Crook, 1993). Cells kept at low temperatures recover more quickly than those with liquid nitrogen storage.

Conclusion

The techniques which I have described above are based on over 25 yr of experience in developing insect cell lines and on many discussions with other researchers in the field. Following these methods will not ensure that you obtain a continuous cell line; an element of chance still exists in this research, which seems to improve as one gains experience in handling cells. However, with patience (remember, it took over a yr after Grace initiated his primary cultures before the cells began to grow consistently) and careful aseptic technique, experience from the past 40 yr has shown that many useful cell lines can be obtained from a wide variety of insects and tissues.

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